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Stabilized Plasmid-Lipid Particles: Pharmacokinetics and Plasmid Delivery to Distal Tumors following Intravenous Injection

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A previous study has shown that plasmid DNA can be encapsulated in lipid particles (SPLP, "stabilized plasmid lipid particles") of approximately 70 nm diameter composed of 1,2-dioleoyl-3-phosphatidyl-ethanolamine (DOPE), the cationic lipid N,N-dioleoyl-N,N-dimethylammonium chloride (DODAC) and poly(ethylene glycol) conjugated to ceramide (PEG-Cer) using a detergent dialysis process (Wheeler *et al.* (1999) Gene *Therapy* 6, 271-281). In this work we evaluated the potential of these SPLPs as systemic gene therapy vectors, determining their pharmacokinetics and the biodistribution of the plasmid and lipid components. It is shown that the blood clearance and the biodistribution of the SPLPs can be modulated by varying the acyl chain length of the ceramide group used as lipid anchor for the PEG polymer. Circulation lifetimes observed for SPLPs with PEG-$CerC_{14}$ and PEG-$CerC_{20}$ were $t_{1/2} = \sim 1$ and ~ 10 h, respectively. The SPLPs are stable while circulating in the blood and the encapsulated DNA is fully protected from degradation by serum nucleases. The accelerated clearance of SPLPs with PEG-$CerC_{14}$ is accompanied by increased accumulation in liver and spleen as compared to PEG-$CerC_{20}$ SPLPs. Delivery of intact plasmid to liver and spleen was detected. Significant accumulation (approximately 10% of injected dose) of the long circulating SPLPs with PEG-$CerC_{20}$ in a distal tumor (Lewis lung tumor in the mouse flank) was observed following iv application and delivery of intact plasmid to tumor tissue at approximately 6% injected dose/g tissue is demonstrated.

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Abbreviations: CHE, cholesteryl hexadecyl ether; Chol, cholesterol; DODAC, N,N-dioleoyl-N,N-dimethylammonium chloride; DOPE, 1,2-sn-dioleoyl-3-phosphatidyl-ethanolamine; POPC, 1-palmitoyl, 2-oleoyl-sn-phosphatidylcholine; EPC, egg phosphatidylcholine; HBS, 20 mM HEPES in 150 mM NaCl pH 7.4; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LUV, large unilamellar vesicle(s); MWCO, molecular weight cutoff; OGP, octylglucopyranoside; PEG-CerC_{14}, 1-0-(Z-(w-methoxypolyethyleneglycol) succinoyl)-2-N-myristoylsphingosine; PEG-CerC_{20}, 1-O-(2'-(ω-methoxypolyethyleneglycol) succinoyl)-2-N-arachidoylsphingosine; PS, phosphatidylserine; SPLP, stabilized plasmid lipid particles; TAE, electrophoresis buffer (40 mM Tris acetate pH 8.5; 2 mM EDTA); RES, reticuloendothelial system; TE buffer, 10 mM Tris–Cl pH 8.0 and 1 mM EDTA

INTRODUCTION

The limitations of the currently available gene delivery systems for systemic application are widely recognized. Viral systems are rapidly cleared from the circulation limiting potential target sites to "first-pass" organs such as lung, liver and spleen. In addition, these systems elicit immune responses compromising the effectiveness of subsequent injections (Worgall *et al., 1997).* The most common non-viral delivery system, the plasmid DNA-cationic lipid complexes (lipoplexes) carry an overall positive charge which results in rapid clearance from the circulation by the reticuloendothelial system (RES) following systemic administration *in vivo* (Mori et *al.,* 1998) limiting potential transfection sites to these "first-pass" organs (Hofland *et al.,* 1997; Lew *et al.,* 1995; Templeton *et al.,* 1997; Thierry *et al.,* 1995; Huang and Li, 1997). Furthermore, lipoplexes tend to form large aggregates ($\geq$ 300nm in diameter) which become trapped in the capillary beds of the lung (Yang and Huang, 1998). Therefore, the highest expression levels are usually observed in the lung (Hofland et *al.,* 1997; Barron et al., 1998; Hong *et al.,* 1997; Felgner *et al.,*1995), while expression levels in other organs are typically more than one magnitude lower. In addition, the bioavailability of the plasmid is compromised since a large amount of the plasmid DNA in lipoplexes is degraded in the blood due to cleavage by serum nucleases (Wheeler *et al.,* 1999).

The characteristics of lipoplexes are potentially useful for gene delivery to lung tissue, for example, but are clearly unsuitable for delivery to a distal tumor site. Pharmacokinetic studies of liposomes have shown that small $\sim$ 100 nm diameter liposomes, which exhibit an extended circulation half-life accumulate preferentially at sites of disease including inflammation, infection and tumors. Such liposomes evade rapid removal from the circulation *in vivo* due to their small size and low surface charge (Allen and Chonn, 1987; Allen *et al.,* 1989; Gabizon and Papahadjopoulos, 1988; 1992). Inclusion of a PEG coating can also enhance the circulation lifetimes considerably. As much as 10% of the injected dose/g of tumor tissue of liposomes with PEG coating has been detected in tumors following systemic application (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1992). This suggests that a small ($\sim$ 100 nm diameter) lipid-DNA particle with a PEG coating, in which the DNA is protected or hidden from the membrane surface and which presents an overall low surface charge should have the characteristics necessary for delivery to a tumor site *in vivo.* In this regard it was recently shown that plasmid DNA can be encapsulated in small stabilized lipid particles (SPLP) by a detergent dialysis method (Wheeler *et al.,* 1999).

Here, we determined the pharmacokinetics of these SPLPs following iv application. Clearance from the blood and biodistribution of SPLPs is shown in two normal mouse strains (CD-1 and BDF-1) and in BDF-1 tumor-bearing mice. The pharmacokinetics can be modulated by varying the lipid composition. Protection of the plasmid in the SPLP from degradation by serum nucleases in

the blood is demonstrated. Accumulation in first-pass organs particularly in the lung is significantly reduced with SPLPs that exhibit circulation longevity. Most importantly it is shown that iv administration of SPLPs results in delivery of high levels of intact plasmid to a distal tumor site.

MATERIALS AND METHODS

Materials

Dioleoylphosphatidylethanolamine (DOPE) was purchased from Avanti Polar Lipids Inc. (AL, USA). The lipids 1-0-(2'-(w-methoxypolyethyleneglycol) succinoyl)-2-N-myristoylsphingosine (PEG-CerC_{14}) and 1-0-(2'-(w-methoxypolyethyleneglycol) succinoyl)-2-N-arachidoylsphingosine (PEG-CerC_{20}) were synthesized as described elsewhere (Webb *et al.*, 1998), and dioleoyldimethylammonium chloride (DODAC) was kindly provided by Dr. S. Ansell (Inex Pharmaceuticals Corp.). ^{3}H and ^{14}C labeled cholesteryl hexadecyl ether (^{3}H-CHE and ^{14}C-CHE) were purchased from Mandel Scientific (Guelph, Ont., Canada). Octylglucopyranoside (OGP), DEAE-Sepharose CL-6B, HEPES and NaCl were obtained from Sigma Chemical Co. (St. Louis, MO, USA). The plasmid pCMVCAT (4490 bp, coding for the chloramphenicol acyl transferase gene) under the control of the human CMV immediate early promoter-enhancer element was originally obtained from Dr. K. Brigham (Vanderbilt University, Nashville, TN, USA). The plasmid was prepared as previously described (Birnboim and Doly, 1979; Sambrook *et al.*, 1989a) and the supercoiled plasmid isolated on a cesium chloride gradient and dialyzed against ultrapure distilled H_2O. The DNA was precipitated and dissolved in pyrogen-free water (1 mg/ml) for formulation with lipids. Mouse serum was obtained from CedarLane (Mississauga, Ont., Canada). Dialysis tubing (SpectraPor 12,000–14,000 mwco) was obtained from Fisher Scientific (Ottawa, Ont., Canada), PicoGreen™ from Molecular Probes (Eugene, OR, USA). All other chemicals were reagent grade.

Preparation of Lipid-DNA Complexes

Vesicles of 100 nm diameter composed of DOPE/DODAC (50 : 50 mol%) were prepared by an extrusionmethod described elsewhere (Hope *et al.*, 1985). Briefly, lipids dissolved in $CHCl_3$ were combined (5mg total lipid) and dried to a lipid film under a stream of nitrogen gas. Any remaining $CHCl_3$ was removed by lyophilization. The lipid was hydrated in 1 ml of HEPES Buffered Saline solution (HBS; 20 mM HEPES, 150 mM NaCl, pH 7.4), subjected to 5 freeze-thaw cycles and extruded through two 100 nm pore diameter polycarbonate filters. Lipid–DNA complexes were then prepared by mixing the appropriate quantity of plasmid (pCMVCAT) with the lipid vesicles to give a charge ratio (±) of 1 : 1.

Preparation and Isolation of SPLPs by Detergent Dialysis

The SPLPs were prepared by a detergent dialysis method as outlined previously (Wheeler *et al.*, 1999). Briefly, DODAC and the "other lipid" species (DOPE, Chol and PEG-ceramide, Cl4 : 0 or C20 : 0) were aliquotted into two separate test tubes from benzene : methanol (95 : 5) stock solutions to give the desired lipid ratio (5 or 10 mg/ml final lipid concentration) and the solvent removed by freeze-drying under high vacuum. Plasmid DNA (200 μg) was added to DODAC dissolved in 500 μl 0.2 M octylglucopyranoside (OGP) in HBS (20 mM HEPES in 150 mM NaCl pH 7.4). The "other lipids" were dissolved in 500 μl 0.2 M OGP in HBS and mixed with the DODAC-DNA suspension. The volume was adjusted to 1 ml with HBS and the mixture was dialyzed against 2 1 HBS for 48 h with two buffer changes. Non-encapsulated plasmid was removed by anion exchange chromatography (DEAE-Sepharose CL-6B column, 1 x 4cm). The sample was eluted with HBS, placed in a dialysis bag and embedded in aquacide to concentrate to the desired volume. Following concentration the sample was dialyzed overnight against HBS to adjust the salt concentration. ^{3}H-CHE, ^{14}C-CHE

and ^{3}H-DNA were added as lipid and plasmid markers where appropriate.

Animal Studies

The SPLP preparations (200 µl) were injected into the lateral tail vein of CD-1 or BDF-1 mice and the animals sacrificed by CO_2 suffocation at the desired time points post-injection. Blood was collected into microtainer tubes following cardiac puncture, organs were harvested and tissues were homogenized by procedures described elsewhere (Parr *et al.*, 1997).

Lewis lung cells were grown in tissue culture using methods described elsewhere (Parr *et al.*, 1997). Approximately 300,000 Lewis lung carcinoma cells (ATCC CRL- 1642) were injected subcutaneously into the left hind flank of BDF-1 mice and tumors were allowed to grow for 14 days. The appropriate formulations (200 µl) were then injected into the lateral tail vein and the animals sacrificed at the desired time points post-injection. Blood collection and tissue harvesting were performed as described above.

Analysis of Lipid and Plasmid Biodistribution

Tissue homogenates were analyzed for ^{3}H-plasmid and ^{14}C-Lipid. Aliquots of tissue homogenates (200 µl of liver; 300 µl of heart, lung and kidney; 400 µl of spleen and 200 µl of tumor as appropriate) were placed in scintillation vials and 500 µl Solvable (Packard, Meriden, CT) added to each vial. Following overnight incubation at 60°C 500 µl distilled H_2O was added to the samples with vortex mixing and the color was bleached by addition of up to 200 µl hydrogen peroxide. Scintillation cocktail (5 ml Picofluor; Packard, Meriden, CT) was added to the sample, incubated at room temperature in the dark overnight and counted the following day. Appropriate corrections were made to account for the lipid and DNA background levels in the vasculature of the various organs.

Determination of Encapsulated DNA by the PicoGreen™ Fluorescence Assay

Plasmid encapsulation was evaluated by measuring the accessibility of the DNA-interchelating dye PicoGreen™ to plasmid at an excitation wavelength of 485nm and emission wavelength of 525nm (Aminco Bowman Series 2 Luminescence Spectrometer, SLM-Aminco, Urbana, IL). Typically 5 µl of PicoGreen was added to 1 ml of sample containing 0.2– 1 .O µg plasmid. Plasmid encapsulation efficiency was calculated as $E(\%) = (I_0 - I)/I_0 \times 100$ where I and I_0 refer to the fluorescence intensities before and after the addition of Triton X-100 (final concentration 0.4%, v/v). Triton X-100 dissolves the SPLP exposing the encapsulated plasmid to the dye. Fluorescence intensities in the absence of PicoGreen were used as background references. The plasmid contents of formulations before and after DEAE column chromatography were determined by this assay. A standard curve was obtained using known quantities of the plasmid of interest in the presence and absence of Triton X-100 (Note, Triton did not affect the fluorescence intensity of the dye.) This assay was linear for up to 1 µg of double-stranded DNA.

Characterization and Quantification of Plasmid DNA in Tissue Extracts

Aliquots (100 µl) of homogenized tissue were added to 0.5 ml DNAzol in an Eppendorf tube, mixed gently by inverting, the tubes were topped up with 95% ethanol and incubated at 4°C overnight. Samples were centrifuged at ~ 10,000 x g for 15 min, the supernatant was discarded and 100 µl of TE was added. The sample was kept at 4°C for 2–3 days with occasional agitation to allow the DNA to dissolve. Ten microliter aliquots of dissolved DNA were analyzed by Southern and dot blot hybridizationmethods according to (Sambrook *et al.*, 1989b).

RESULTS

Formulations

Two SPLP formulations with different lipid composition were made. The major lipid constituents were POPC and cholesterol (PC/Chol-SPLP) or the fusogenic lipid DOPE (DOPE-SPLP). In both cases the particles were stabilized with 10% PEG-ceramide. Particles were characterized for their size and encapsulation efficiencies achieved. To obtain high plasmid entrapment with the different lipid compositions adjustments in the cationic lipid concentration were necessary. Optimal encapsulation was observed for PC/Chol SPLPs (40–50% plasmid encapsulation) with 12% DODAC (DODAC/POPC/Chol/PEG-CerC_{20}, 12/38.7/39.8/ 9.5 mol%) and for DOPE-SPLPs (60–70% encapsulation) with 6% DODAC (DODAC/DOPE/ PEG-CerC_{20}, 6/84/ 10 mol%). The particle size in both formulations was approximately 100 nm with 75 ± 34 nm for DOPE-SPLPs and 118 ± 45 nm for PC/Chol–SPLPs as measured by dynamic light scattering. Further characterizations of SPLPs are outlined in detail elsewhere (Wheeler *et al.*, 1999).

Plasmid Protection in SPLPs

An important aspect of a delivery system designed for systemic application is its ability to retain its payload in an intact form during circulation. The integrity of the SPLPs was tested by determining the protection of the plasmid from degradation by serum nucleases. The different preparations (free plasmid, plasmid complexed with DOPE/DODAC LUVs, DOPE-SPLP and POPC/Chol-SPLP) were incubated in 90% mouse serum at 37°C for up to 5 h and the plasmid DNA characterized by Southern hybridization analysis. The percentage of intact DNA as a function of incubation time is shown in Fig. 1. Over 80% of the encapsulated plasmid in both SPLP preparations remained intact following a 5 h incubation period. However, free plasmid and plasmid complexed to DODAC/DOPE LUVs was degraded completely within 1 h.

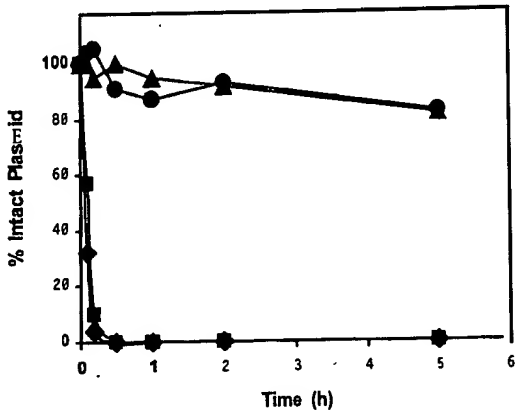

FIGURE 1 Protection of plasmid in DOPE-SPLP and POPC/Chol–SPLP from serum nucleases. Free plasmid (◆), plasmid complexed with DODAC/DOPE vesicles (■), DOPE-SPLP (●) and POPC/Chol-SPLP (▲) was incubated in 90% (v/v) normal mouse serum at 37°C for the time intervals as indicated. Following incubation the plasmid was extracted using DNAzol and characterized by Southern hybridization analysis. Data are presented as percentage of intact DNA remaining (time 0= 100%) and plotted as a function of incubation time.

Clearance and Biodistribution of SPLPs *In vivo* is Modulated by the PEG Anchor

Considerable circulation retention *in vivo* is necessary in order to achieve accumulation of SPLPs in a distal tumor (Gabizon and Papahadjopoulos, 1988). Previous studies have shown that circulation longevity can be achieved with liposomes coated with PEG (Parr *et al.*, 1997). However, the presence of the PEG coating is expected to inhibit the association and fusion of the SPLPs with cells in a similar manner to the inhibition of fusion between LUVs by PEG coatings (Holland *et al.*, 1996). In that study it was shown that LUVs containing PEG linked to a lipid anchor with short acyl chains that permits exchange of PEG out of the LUVs, will become increasingly fuogenic with the loss of PEG. Here, we studied the effect of PEG anchored to ceramide of different acyl chain lengths (CerC_{14} and CerC_{20}) on the clearance of the SPLP. As shown in Fig. 2(a) POPC/Chol–SPLPs containing PEG-CerC_{20} exhibit an extended circulation lifetime

($t_{1/2} \sim 10$ h) whereas SPLPs with PEG-CerC_{14} are cleared rapidly ($t_{1/2} \sim 1$ h). The clearance profile observed for PE–SPLPs with PEG-CerC_{14} (data not shown) and PEG-CerC_{20} (Fig. 4(a)) were similar to the corresponding PC/Chol–SPLPs.

The different circulation longevity of the SPLPs is reflected in the lipid accumulation in the organs as summarized in Table I. Particles with the shorter hydrophobic anchor PEG-CerC_{14} are cleared mainly by the liver which accounts for $\sim$ 50% of the injected dose at the 1 h time point. Accumulation in the spleen at this time is approximately 2% of injected dose. Only minute levels of the lipid marker were detected in lung, heart and kidney. For particles with PEG-CerC_{20} approximately 20% and 1% of the injected lipid dose appeared in the liver and spleen respectively, at the 1 h time point. Again negligible amounts of the labeled lipids were detected in other organs.

Integrity of Plasmid in SPLP in the Circulation

The clearance properties of the SPLPs based on a lipid marker alone do not provide information about the stability of the particle and its payload. To evaluate the integrity of the plasmid inside the

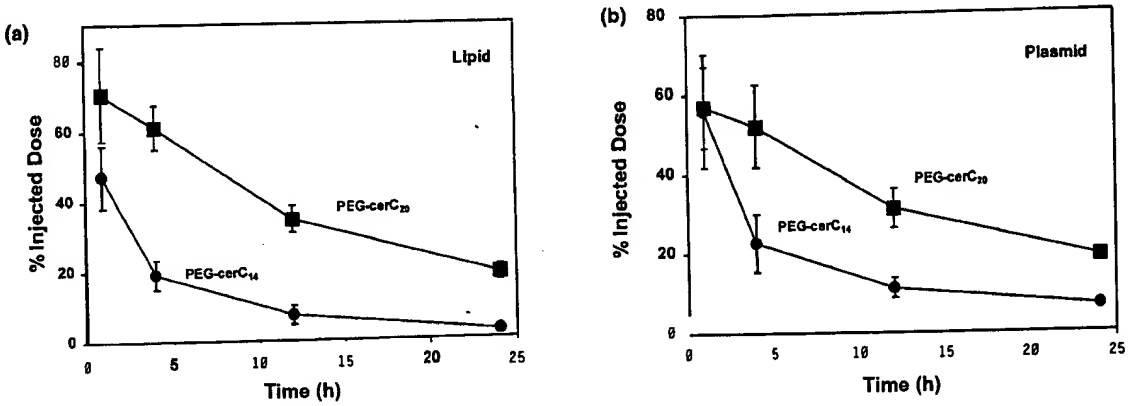

FIGURE 2 Clearance of POPC/Chol-SPLP stabilized with PEG-CerC_{14} and PEG-CerC_{20} from blood following iv injection. POPC/Chol-SPLP with PEG-CerC_{14} (●) and POPC/Chol-SPLP with PEG-CerC_{20} (■) were injected into CD-1 mice at a dose of 30 μg plasmid and $\sim$ 1 mg ^{3}H-lipid. Mice were sacrificed 1, 4, 12 and 24 h following injection, Plasma samples were analyzed for radioactivity (^{3}H-lipid) by scintillation counting and for intact plasmid by Southern hybridization analysis as described in Methods. Data are expressed as percent injected dose of lipids (a) and plasmid (b) remaining in the plasma and plotted as a function of time. The plasmid dose was estimated based on encapsulation efficiency.

TABLE I Biodistribution of PC/Chol-SPLP in CD-1 mice

Lipid–DNA particle	Time post-injection (h)	% Injected dose					
		Plasma (SD)	Spleen (SD)	Liver (SD)	Lung (SD)	Heart (SD)	Kidney (SD)
SPLP–CerC_{20}	1	70.5 (13.2)	0.96 (0.78)	21.23 (8.21)	0.29 (0.34)	0.58 (0.19)	-0.06 (0.08)
	4	61.02 (6.32)	0.74 (0.04)	18.78 (4.01)	0.38 (0.39)	0.86 (0.20)	0.46 (0.16)
	12	34.39 (3.88)	0.96 (0.34)	18.83 (1.38)	0.32 (0.04)	0.39 (0.06)	0.77 (0.26)
	24	18.46 (2.14)	1.5 (0.19)	23.37 (6.16)	0.21 (0.26)	0.25 (0.12)	1.31 (0.14)
SPLP–CerC_{14}	1	47.18 (8.84)	1.22 (0.8)	48.03 (2.13)	1.13 (0.48)	0.44 (0.28)	0.36 (0.25)
	4	18.93 (4.19)	2.18 (0.77)	56.87 (4.7)	0.55 (0.10)	0.18 (0.04)	0.50 (0.22)
	12	7.20 (2.68)	2.07 (0.39)	48.61 (12.59)	0.37 (0.07)	0.18 (0.06)	0.66 (0.20)
	24	2.17 (0.84)	1.99 (0.46)	55.39 (7.59)	0.39 (0.10)	0.14 (0.05)	0.66 (0.05)

particles during circulation, plasmid DNA recovered from blood samples at various times following injection were analyzed by Southern blot hybridization methods. The amount of intact plasmid decreases over time and as shown in Fig. 2(b), the plasmid clearance profile reflects the lipid clearance from the blood. Intact plasmid was detected in the plasma of mice injected with PC/Chol–SPLPs with PEG-CerC_{14} and PEG-CerC_{20} even 24 h after administration (see Fig. 3). This clearly indicates that plasmid is retained in the SPLPs and is fully protected from degradation by serum nucleases.

The DOPE-SPLPs exhibit similar stability and plasmid protection as PC/Chol-SPLP (Fig. 4). In this case, radiolabeled lipid (^{14}C-CHE) and DNA (^{3}H-Plasmid) were used to follow the fate of the respective components. The DNA to lipid ratio in the samples remained constant (~ 1) at all time points (Fig. 4(b)) suggesting stable particles and retention of plasmid in the circulation. Southern hybridization analysis confirmed the integrity of the DNA (see below for BDF-1 mice, Fig. 6).

DOPE-SPLP Biodistribution and Delivery of Intact Plasmid to Organs

The tissue accumulation of DNA and lipid from DOPE-SPLPs with PEG-CerC_{20} is summarized in Table II. We find that both lipid and plasmid levels in the spleen are consistently low. The amount of lipid detected in the liver increases slowly over the 24 h period (24% of injected dose after 24 h),

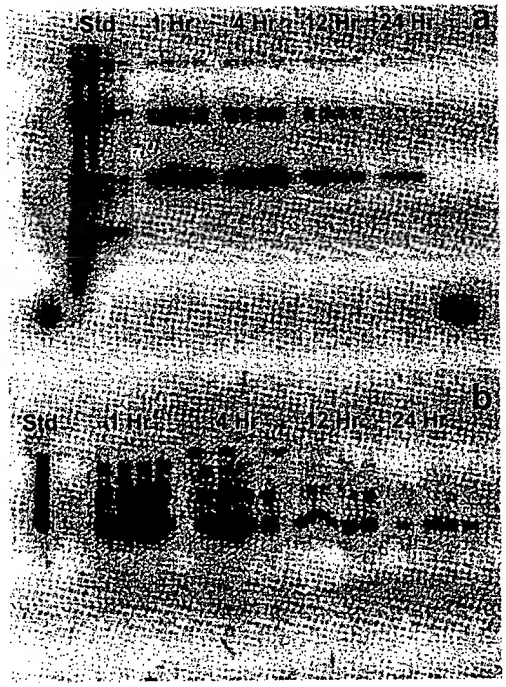

FIGURE 3 Integrity of plasmid in the SPLP recovered from the blood after iv injection of SPLP. Southern hybridization analysis of plasmid DNA recovered from the blood of CD-1 mice 1, 4, 12 and 24 h following injection of (a) POPC/Chol–SPLP with PEG-CerC_{20} and (b) POPC/Chol–SPLP with PEG-CerC_{14}. The plasmid was recovered from plasma samples using DMAzol and assayed by Southern hybridization.

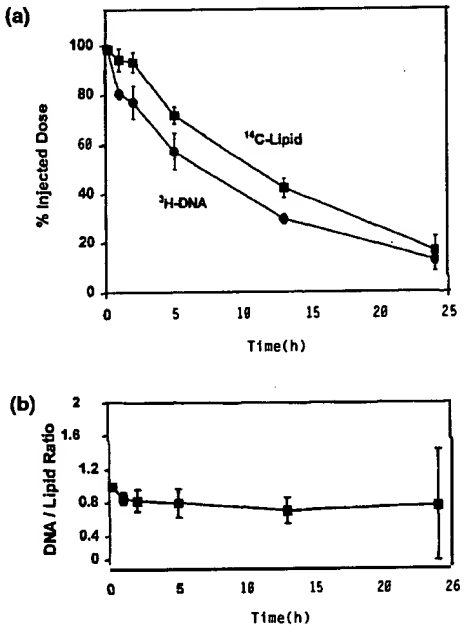

FIGURE 4 Clearance of DOPE-SPLP from blood following iv injection. DOPE-SPLP (2 mg ^{14}C-lipid; 30 μg ^{3}H-DNA) was injected into CD-1 mice. Mice were sacrificed 5 min, 1, 2, 5, 13 and 24 h after injection. Plasma samples were analyzed for radioactivity (^{3}H-DNA and ^{14}C-lipids) by scintillation counting. Data are expressed as percent injected dose of lipids (■) and plasmid (●) remaining in the plasma and plotted as a function of time in (a). The DNA to lipid ratio (% dose/% dose) as a function of time is plotted in (b).

TABLE II Biodistribution of [^{3}H]-DNA encapsulated in [^{14}C]-DOPE–SPLP ($CerC_{20}$) in CD-1 mice

Time	Marker	% Injected dose						
		Blood	Spleen	Liver	Lung	Heart	Kidney	Total
5 min	[^{3}H]	98.7 (1.2)	0.0 (0.0)	1.5 (0.0)	0.1 (0.3)	1.9 (0.1)	0.0 (0.0)	102.0 (0.5)
	[^{14}C]	98.7 (1.0)	0.0 (0.0)	0.1 (0.4)	0.3 (0.1)	1.9 (0.0)	0.0 (0.0)	101 .o (0.7)
	[^{3}H]/[^{14}C]	1.000 (0.003)						
1 h	[^{3}H]	80.6 (2.0)	0.4 (0.6)	2.6 (0.4)	0.6 (0.1)	1.2 (0.1)	0.2 (0.5)	85.7 (2.1)
	[^{14}C]	94.4 (4.7)	0.0 (0.2)	3.7 (0.1)	0.5 (0.1)	1.2 (0.1)	0.0 (0.2)	99.6 (4.9)
	[^{3}H]/[^{14}C]	0.855 (0.028)						
2 h	[^{3}H]	17.3 (6.7)	1.2 (0.9)	2.9 (1.4)	0.5 (0.3)	1.2 (0.3)	0.2 (0.3)	83.3 (6.7)
	[^{14}C]	93.4 (4.1)	0.0 (0.2)	5.9 (1.1)	0.7 (0.2)	1.1 (0.3)	0.0 (0.0)	101.0 (5.1)
	[^{3}H]/[^{14}C]	0.827 (0.039)						
5 h	[^{3}H]	57.4 (7.2)	1.3 (0.2)	2.6 (1.3)	0.5 (0.2)	0.8 (0.3)	0.7 (0.3)	63.2 (6.6)
	[^{14}C]	71.9 (3.5)	0.3 (0.2)	9.0 (2.0)	0.8 (0.3)	0.8 (0.3)	0.3 (0.3)	83.2 (3.4)
	[^{3}H]/[^{14}C]	0.797 (0.078)						
13h	[^{3}H]	30.0 (1.9)	1.3 (0.2)	3.0 (0.5)	0.4 (0.1)	0.6 (0.3)	1.2 (0.5)	36.5 (2.0)
	[^{14}C]	42.6 (4.0)	1.3 (0.1)	18.0 (2.2)	0.7 (0.1)	0.6 (0.2)	1.6 (0.4)	64.8 (2.0)
	[^{3}H]/[^{14}C]	0.706 (0.023)						
24h	[^{3}H]	13.2 (4.4)	1.1 (0.2)	3.5 (0.2)	0.5 (0.1)	0.5 (0.0)	1.4 (0.2)	20.2 (4.6)
	[^{14}C]	17.1 (5.7)	2.1 (0.2)	23.5 (2.4)	0.6 (0.1)	0.5 (0.1)	3.1 (0.7)	46.9 (3.0)
	[^{3}H]/[^{14}C]	0.768 (0.004)						

however, the amount of plasmid detected at all time points remains at approximately 3-4% of the injected dose. This probably reflects the relative rates of degradation of the ^{3}H-plasmid and ^{14}C-CHE in the liver over time. To investigate this possibility further we characterized the plasmid DNA extracted from the liver and spleen. For this study SPLPs with PEG-$CerC_{14}$ were chosen since they showed the highest accumulation in the liver and spleen. Tissues derived from animals treated with PC/Chol–SPLPs containing PEG-$CerC_{14}$ were examined by Southern hybridization analysis (Fig. 5(a)). Clearly, significant quantities of intact plasmid were detected in the liver and spleen at all time points. In the liver, plasmid DNA appears to be degraded very quickly over time while in the spleen the degradation occurs at a much slower rate (Fig. 5(b)). This is consistent with the results from the SPLPs labeled with radioisotopes only shown above. A similar plasmid distribution was also observed for DOPE-SPLPs with PEG-$CerC_{14}$, thus the Southern Blot and the DNA profile shown in Fig. 5 are representative of the fate of plasmid DNA delivered by the SPLPs.

Pharmacokinetics of SPLPs in Normal and Tumor-Bearing BDF-1 Mice

The murine fibrosarcoma line and mouse model were chosen largely for the high degree of vascularization of the tumor *in vivo* and since tumor propagation in BDF-1 mice has been shown previously to be highly successful (Harasym *et al.*, 1997). SPLPs with PEG-$CerC_{20}$ were used for these studies as they exhibited the most extended circulation lifetimes (Figs. 2 and 4). The pharmacokinetics for DOPE-SPLP and PC/Chol-SPLP with PEG-$CerC_{20}$ were determined in non-tumor bearing and tumor-bearing BDF- 1 mice and compared to that of free plasmid and plasmid complexed to DODAC/DOPE LUVs.

In normal BDF- 1 mice the SPLP are cleared from the circulation at a similar rate as in CD-1 mice. As an example, the clearance profile for DOPE-SPLPs is shown in Fig. 6. Blood samples were analyzed for intact plasmid at different time intervals following injection by Southern hybridization analysis. As observed previously in CD-1 mice (Fig. 4) plasmid and lipid were removed from the blood

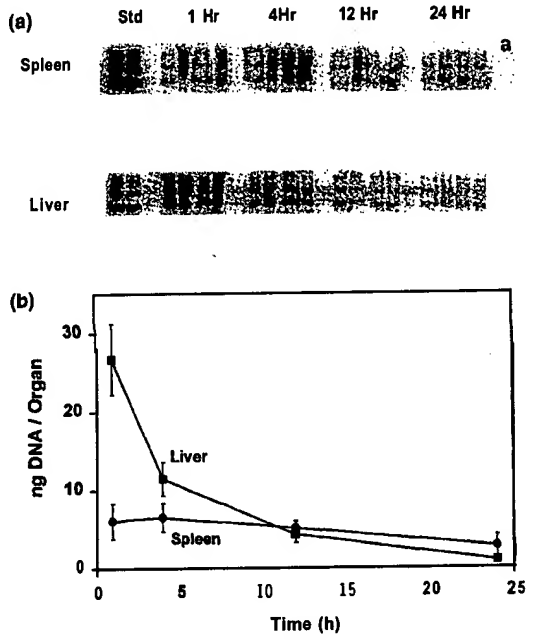

FIGURE 5 Southern hybridization analysis of plasmid DNA extracted from spleen and liver. CD-1 mice were injected iv with POPC/Chol–SPLP with PEG-CerC_{14} and sacrificed 1, 4, 12 and 24 h post-injection. Plasmid was extracted from spleen and liver homogenates and analyzed by Southern hybridization (a). In (b) the amount of intact plasmid recovered in liver (■) and spleen (●) is plotted for different time points following injection.

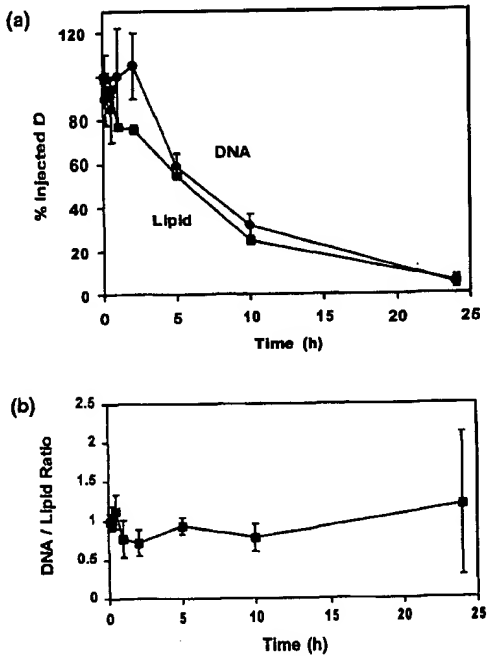

FIGURE 6 Plasma clearance of DOPE-SPLP in BDF-1 mice. DOPE-SPLP was injected into normal BDF-1 mice at 30 μg plasmid DNA per mouse. Mice were sacrificed at 0.1, 0.2, 0.5, 1, 2, 5, 10, 24 h after injection. Plasma samples were analyzed for ^{3}H-labeled lipid by scintillation counting. Plasmid DNA was recovered from the plasma using DNAzol and assayed by Southern hybridization analysis. Data are expressed as percent of injected dose of lipid (■) and intact plasmid (●) remaining in the plasma and plotted as a function of time after injection (a). Bars represent SD (n = 3). The DNA to lipid ratio (% dose/% dose) as a function of time is plotted in (b).

concomitantly. The biodistribution for DOPE–SPLPs and PC/Chol–SPLPs are summarized in Table III for the 1 and 24 h time points post-injection and compared to DODAC/DOPE–plasmid complexes and free plasmid. Accumulation in the liver and spleen are listed separately while for the lung, heart and kidney it is combined together as "others" since the SPLP accumulation in these organs was consistently minimal. As was expected, the DODAC/DOPE–plasmid complexes were cleared almost completely from the blood, primarily by the liver, within 1 h following injection. The two encapsulated formulations, however, maintained their integrity in the blood with approximately 60% and 86% of PC/Chol-SPLP and DOPE–SPLP, respectively, remaining after 1 h and between 10% and 20% after 24h with approximately an equivalent amount of intact plasmid.

Delivery of Intact Plasmid to a Subcutaneous Lewis Lung Carcinoma

The pharmacokinetics of the DOPE–SPLPs in BDF- 1 mice bearing a Lewis lung carcinoma in the left hind flank (Fig. 7(a)) appears to be similar to that in non-tumor bearing animals. Accumulation of remarkably high levels of lipid (10% of injected dose) and intact plasmid (6% of injected dose) were observed in the tumor over the first 10 h following bolus injection into the lateral tail vein (Fig. 7(b)). Approximately 4% of the plasmid dose was still detected 24 h following injection.

The accumulation of lipid and plasmid of DOPE–SPLPs in tumor was compared to the accumulation obtained with PC/Chol–SPLPs and

TABLE III Biodistribution of different liposomal formulations with plasmid DNA in normal BDF-1 mice[a]

Plasmid formulation [b]	Time (h)	% Injected dose[a]				
		Plasmid DNA	^{3}H-Lipid			
		Plasma	Plasma	Liver	Spleen	Others[d]
(A) Free plasmid DNA	1	2.4 (1.3)	n/a	n/a	n/a	n/a
	24	1.5 (0.4)	n/a	n/a	n/a	n/a
(B) DOPE/DODAC LUVs	1	2.3 (0.9)	3.2 (1.3)	43.5 (0.4)	4.1 (0.6)	3.7 (0.4)
	24	2.8 (0.2)	3.3 (2.5)	45.8 (3.5)	4.1 (1.0)	2.4 (0.3)
(C) DOPE/DODAC/PEG-Cer (C_{20}) SPLP	1	82.9 (6.9)	85.9 (2.1)	2.7 (0.3)	0.3 (0.0)	1.7 (0.2)
	24	15.7 (0.3)	11.0 (3.3)	23.0 (2.3)	2.1 (0.1)	1.6 (0.1)
(D) POPC/Chol/DODAC/PEG-Cer (C_{20}) SPLP	1	69.3 (27.2)	59.5 (6.0)	21.0 (1.5)	1.9 (0.3)	1.6 (0.4)
	24	14.5 (2.2)	23.2 (6.0)	29.4 (5.4)	2.2 (0.8)	1.6 (0.4)

[a]Dose: 30 μg plasmid DNA and ~1–2 mg lipid per mouse. [b]pINEXCAT plasmid DNA was either complexed with LUVs (B) or encapsulated in SPLP (C) and (D). [c]Data are expressed as the percentage of injected dose of lipid or plasmid DNA together with SD (n = 3). DNA was determined by Southern blot analysis and lipid by the radiolabel ^{3}H-CHE. [d]Others include lungs, heart and kidneys.

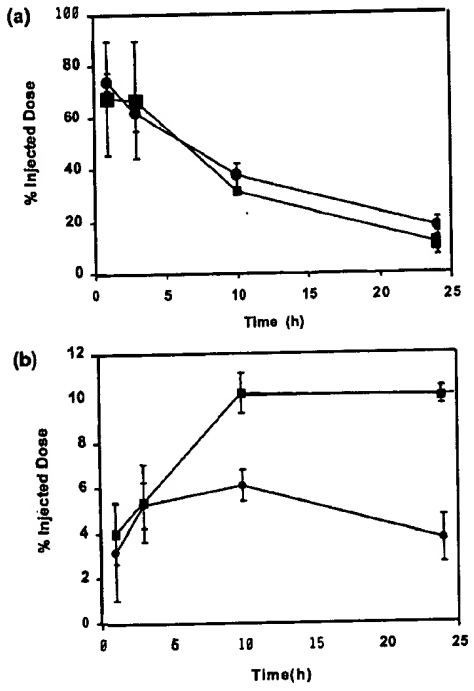

FIGURE 7 Tumor accumulation of and plasma clearance of DOPE-SPLP in BDF-1 mice bearing Lewis lung tumor. Mice were seeded with tumor cells and after 14 days injected with DOPE-SPLP at a dose of 30 μg plasmid DNA and ~ 2mg lipid. Animals were sacrificed 1, 3, 10 and 24h post-injection. Plasma samples (a) and tumor tissue (b) were analyzed for ^{3}H-lipids by scintillation counting and for intact plasmid by Southern hybridization analysis. The amount of lipid (■) and intact plasmid (●) recovered from tumor tissue and blood are given as percent injected dose and plotted as a function of time following injection.

DODAC/DOPE–plasmid complexes (Table IV). The amount of intact plasmid detected in the tumor for PC/Chol–SPLPs (4.4% ± 0.7%) 10 h post-injection was not significantly different to that for DOPE-SPLPs (6.1% ± 0.7%). Only trace amounts of plasmid (< 0.1%) were detected in the tumor following injection of DOPE/DODAC–plasmid complexes. The percentage of SPLPs remaining in the circulation 10 h post-injection was lower for PC/Chol–SPLPs. However, this is likely due to differences in the lipid dose injected. The lipid dose was lower for the PC/Chol–SPLPs than for the DOPE-SPLPs and probably resulted in a somewhat faster clearance, similar to the liposomal clearance rates previously reported for different lipid doses (Oja *et al.*, 1996). The apparent accumulation of plasmid and lipid diverge over time and could reflect the processing of the SPLP in tumor tissue indicating a faster metabolism of the plasmid as compared to the lipid.

DISCUSSION

The primary objective of this study was to develop a gene delivery system for systemic application that results in preferential accumulation at disease sites such as inflammation or tumor. It has been demonstrated previously that the delivery of anti-cancer

TABLE IV Tumor and plasma levels of different liposomal formulations of plasmid DNA following iv injection[a]

Plasmid formulation[b]	% Injected dose[c]			
	Tumor		Plasma	
	Plasmid DNA	Lipid	Plasmid DNA	Lipid
(A) Free plasmid DNA	0.1 (0.3)	n/a	0.0 (0.4)	n/a
(B) DOPE/DODAC LUVs	0.1 (0.4)	1.1 (0.2)	0.0 (0.3)	2.5 (0.3)
(C) DOPE/DODAC/PEG-Cer (C20) SPLP	6.1 (0.7)	10.2 (0.9)	37.9 (4.5)	31.5 (1.1)
(D) POPC/Chol/DODAC/PEG-Cer (C20) SPLP	4.4 (0.7)	5.3 (0.7)	9.9 (3.7)	14.6 (1.3)

[a]50 μg plasmid DNA was injected per mouse as free plasmid or liposomal formulations as indicated. Mice were sacrificed 10 h following injection. Tumor tissues and plasma samples were analyzed for plasmid DNA by Southern hybridization analysis and for lipid by scintillation counting.
[b]pINEXCAT plasmid DNA was either complexed with LUVs (B) or encapsulated in SPLP (C) and (D).
[c]Data are expressed as the percentage of injected dose of liposomes or plasmid DNA together with SD ($n = 3$). Data for tumor tissues are presented as percentages of injected dose per gram tissue.

drugs to tumor tissue can be achieved with carrier systems where the drug of interest is encapsulated in 100 nm diameter liposomes (LUVs). These vesicles evade uptake by the RES resulting in extended circulation lifetimes and have been shown to accumulate at tumor sites (Gabizon and Papahadjopoulos, 1988; 1992; Allen and Hansen, 1991). The increased accumulation was attributed to enhanced permeability of the tumor vasculature allowing extravasation of small particles into the surrounding tissue (Wu *et al.*, 1993; Hobbs *et al.*, 1998; Monsky et *al.*, 1999). Based on these observations, the following requirements are expected for a lipid based plasmid delivery system targeting these disease sites: (I) extended circulation lifetime, (II) small size of approximately 100 nm, (III) highly stable particles providing full protection of the plasmid from degradation by serum nucleases, (IV) particles able to interact with and enter into target cells upon arrival at the disease site and (V) efficient intracellular delivery of plasmid.

Presently, the favored delivery systems for gene transfer are viral systems since they best address the latter two requirements. The engineered viruses are efficient at transferring foreign genes into cells, however, they are highly immunogenic and consequently of limited use particularly for repeat systemic application. The most widely used non-viral gene delivery systems are DNA-lipid complexes formed by mixing plasmid DNA with lipid vesicles (SUVs or LUVs) composed of a cationic lipid derivative and DOPE as a helper lipid. The complexes formed are capable of transporting the plasmid across cell membranes achieving delivery into a wide spectrum of cells *in vitro*, a process known as lipofection. The major drawbacks of these systems are their tendency to aggregate in serum, the short circulation lifetime and the limited protection of the plasmid from degradation by serum nucleases (Fig. 1) (Lew *et al.*, 1995; Mahato *et al.*, 1995). The fast clearance of the DNA-lipid complexes from the blood is not surprising since charged particles are known to bind serum proteins targeting them for removal by the phagocytic cells of the RES (Allen *et al.*, 1984; Illum and Davis, 1983; Chonn *et al.*, 1991; 1992; Semple *et al.*, 1998). Furthermore, large complexes and aggregates can be trapped in the lung capillaries. The latter will result in an extended exposure of the lung endothelial bed to DNA-lipid complexes and is most likely the reason for the preferential transfection observed in the lung following iv administration of these complexes (Templetor. *et al.*, 1997; Liu *et al.*, 1997).

This paper addresses the first three requirements; of a plasmid delivery system to access disease sites as discussed above. The strategy was to encapsulate, the DNA inside a lipid shell forming DNA-lipid particles of ~100 nm diameter in order to achieve extended circulation lifetimes as well as protection of the plasmid DNA. The PEG-lipid derivatives included in our SPLP play a key role in attaining these desired features. Initially the PEG lipid

stabilizes the particles containing a high concentration of the fusogenic lipid DOPE. Following injection into the blood stream PEG shields the positively charged lipid DODAC, required for efficient encapsulation, from interactions with serum proteins known to cause rapid clearance of lipid vesicles (Semple *et al., 1998).* LUVs with GM1 (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988) or PEG-PE (Allen *et al.,* 1991; Parr *et al., 1994)* amphiphiles included in the membrane were shown to exhibit extended circulation times by successfully avoiding uptake by the RES. A potential drawback of the polymer coating of the particles is a reduced particle-cell surface interaction, which is necessary to induce cellular uptake. Therefore, a polymer coating was designed that dissociates from the particles over time. It was expected that these polymer-coated carriers could initially evade uptake by the RES resulting in prolonged circulation lifetime and leading to accumulation at disease sites. Following exchange of the polymer from the carrier, cellular uptake can then occur. Inclusion of PEG-PE in these SPLPs was not considered, primarily because its negative charge interferes with DNA encapsulation and also based on previous results, which indicate that the exchange rate of PEG-PE from liposomes is too slow (Mori *et al.,* 1998) presumably due to electrostatic attraction to the cationic lipid DODAC. Employing a neutral PEG-lipid circumvented these problems. Ceramide was chosen as the lipid anchor since the anchor strength can be varied by using a ceramide backbone with different acyl chain lengths. An increase in the acyl chain length of the ceramide will increase the hydrophobicity of the anchor resulting in a lower exchange rate of PEG as shown in an *in vitro* model system (Holland *et al.,* 2000). Previously we have shown that the transfection potential of the SPLP *in vitro* is indeed dependent on the depletion of the PEG-coating. SPLP with PEG-$CerC_{20}$, which has a long residence time in the lipid bilayer, showed low transfection whereas greatly improved transfection was obtained with SPLP containing PEG-$CerC_{14}$ that dissociates much more rapidly (Wheeler *et al.,* 1999).

In this study two ceramide anchors were evaluated ($CerC_{14}$ and $CerC_{20}$). The clearance of the SPLP is evidently related to the PEG anchor used as shown in Fig. 2. Circulation longevity was achieved with SPLPs containing PEG-$CerC_{20}$ while SPLPs with PEG-$CerC_{14}$ are cleared from the blood much more rapidly. This is consistent with the accelerated exchange rate observed for the shorter PEG ceramide anchor C_{14} compared to that of C_{20} with $t_{1/2}$ of 1.2 hand several days, respectively (Wheeler *et al.,* 1999) and demonstrates the possibility of modulating the circulation lifetime of the SPLP. The SPLPs are stable in the blood and protect the plasmid during circulation as indicated by the observed plasmid-to-lipid ratio of approximately 1 detected in blood samples recovered after different time intervals following injection for SPLP with PEG-$CerC_{14}$ and PEG-$CerC_{20}$, respectively (Figs. 4(b) and 6(b)). The virtually complete protection of the plasmid in the SPLP during circulation in the blood is a key feature of these particles and was not obtained with any of the previously described lipid carriers.

The SPLPs are cleared from the blood mainly by the liver since the accumulation in this organ of 50% and 20% 1 h after injection for SPLP with PEG-$CerC_{14}$ and PEG-$CerC_{20}$, respectively, mirrors the clearance of these particles (Table I). The liver accumulation values for both the PC/Chol and DOPE-based SPLPs with the PEG-$CerC_{20}$ derivative in CD-1, BDF-1 and BDF-1-tumor-bearing mice are very similar to those attained previously with liposomes containing PEG-PE (Allen et al., 1991; Parr *et al.,* 1994) or GM1 (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; 1992). Note that there was no significant accumulation of any of the SPLPs in the lung as predicted for particles of this size. Importantly, intact plasmid was delivered by the SPLP to liver and spleen and the slow degradation observed over time is suggestive of the bioavailability of the plasmid at the site.

Finally, the SPLPs with extended circulation lifetimes show significant accumulation in Lewis Lung Carcinoma tissue following iv administration and delivery of intact plasmid (6% of injected dose).

The lipid accumulation detected in tumor tissue (approximately 10% of injected dose) is similar to the highest levels previously reported (6-10%) for liposomes that exhibit comparable pharmacokinetics to the SPLP (Allen and Chonn, 1987; Gabizon and Papahadjopoulos, 1988; 1992; Allen *et al.*, 1991; Parr *et al.*, 1994). These data confirm that circulation longevity is indeed an important parameter for a carrier system to deliver plasmid successfully to tumor tissue. The accumulation of intact plasmid in the tumor mass is clearly substantial, but the level of gene expression observed in tumor tissue was low (data not shown). However, the long residence times (10–24 h) of the SPLPs in tumor tissue following the bolus injection indicates that the SPLPs did extravasate into the interstitial space of the tumor and are available for uptake by tumor cells. The accelerated decrease of intact plasmid in the tumor tissue as compared to the lipid indicates processing of the SPLPs in the tumor tissue and bioavailability of the plasmid. The released plasmid is broken down faster by nucleases than the lipid marker is metabolized. Most likely, the SPLPs are taken up preferentially by phagocytic host-cells and macrophages. Incorporation of ligands into the SPLP for specific and increased uptake by tumor cells might be required.

In summary, the SPLP described here fulfill the first three requirements for a plasmid delivery system outlined earlier. We have demonstrated delivery of intact plasmid to a distal tumor site with a lipid based carrier system following systemic administration. The plasmid is encapsulated inside a lipid bilayer and therefore fully protected from degradation. The dissociable polymer coating of the SPLPs allows the particles to avoid the clearance by the RES leading to accumulation in tumor tissue. The delivery of intact plasmid into tumor tissue represents the first step in the development of an efficient gene carrier system with application in cancer gene therapy. Presently, the cellular uptake of the carrier at the disease site, identification of cell types and the fate of the plasmid are evaluated as critical steps towards the final goal, the efficient expression of a desired gene.

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